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(71) Applicant: LIGAND PHARMACEUTICALS, INC. [US/US]; 9393 Towne Centre Drive, San Diego, CA 92121 (US).

(72) Inventors: HERMANN, Thomas; 950 Santa Helena Park Court, Solana Beach, CA 92075 (US). PIKE, John, W.; 911 Springwood Lane, Encinitas, CA 92024 (US).

(74) Agents: WARBURG, Richard, J. et al.; Lyon & Lyon, First Interstate World Center, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).

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(54) Title: SCREENING FOR CYTOKINE MODULATORS

(57) Abstract

This invention provides a method for screening for agents useful for treatment of diseases and pathological conditions affected by cytokines. These agents interact directly or indirectly with an intracellular receptor, which in turn modulates the binding of a rel-like protein, a rel-like protein complex, or other transcriptional proteins to a rel site on the promoter of a cytokine gene. The intracellular receptor can be the estrogen receptor, retinoid acid receptors, retinoid X receptors, glucocorticoid receptor, progesterone receptors, androgen receptor, thyroid hormone receptors, or vitamin D receptor. The select agents can be used to treat osteoporosis, rheumatoid arthritis, inflammation, psoriasis, Kaposi's sarcoma, septic shock and multiple myeloma.

SCREENING FOR CYTOKINE MODULATORS

FIELD OF THE INVENTION

This invention relates to a method for screening for agents useful for treatment of diseases and pathological conditions affected by cytokines and novel agents identified using such screening method.

BACKGROUND OF THE INVENTION

Cytokines are a group of molecules capable of signalling cellular development. Aberrant expression of cytokines is known to be associated with pathological conditions including autoimmune diseases, septic shock, rheumatoid arthritis, psoriasis, inflammation, postmenopausal osteoporosis, and some cancers. Common treatment for these pathological conditions are retinoids, immunosuppressants, glucocorticoids and other steroid drugs. Estrogens are specifically employed in the prevention of postmenopausal osteoporosis.

Steroids and related hormone drugs exert their therapeutic effects by binding to a superfamily of intracellular receptors (IRs), which are regulators of gene transcription. IRs can function as activators as well as repressors of specific cytokine genes. The activity of IRs is controlled by hormones or other ligands that bind to the IRs.

The classical mechanism of transcriptional regulation by IRs involves binding of the IRs to specific response elements in the promoters of the regulated genes, for example, the binding of the estrogen receptor to its response site in the vitellogenin gene (Klein-Hitpass et al., Cell 46:1053-1061, 1986). More recently a different mechanism of IRs function has been described in glucocorticoid receptor mediated AP-1 transcription regulation that does not

the control of the transcriptional activity of NFκB or closely related proteins on the IL-6 promoter. This mechanism does not involve direct binding of ER to IL-6 promoter but controls the DNA-binding properties of the activated NFκB and possible other members of the rel-family of proteins to their specific response elements (i.e., rel site) on the IL-6 promoter.

Because NFκB is involved in the regulation of genes encoding various cytokines and their receptors, viral proteins, and proteins involved in the acute-phase response, the regulation of NFκB activity by estrogen and possible other hormones is of general importance (see generally Baeuerle, Biochemica et Biophysica Acta, 1072:63-80, 1993, incorporated by reference herein). For example, retinoic acid treatment, which strongly inhibits IL-6 expression in +/+LDA11 cells and other tissues (Gross, V., P. M. Villiger, B. Zhang, and M. Lotz, 1993, "Retinoic acid inhibits interleukin-1-induced cytokine synthesis in human monocytes," J. Leukoc. Biol. 54:125-132), has the same effect as estrogen on the NFκB related complexes with the IL-6 promoter. This suggests a general pathway of transcriptional regulation involving cross-talk between members of the intracellular receptor family and the NFκB transcription factors.

The above determination allows for the screening of drugs that specifically influence genes controlled by the rel-transcription factors, i.e. genes involved in inflammation, sepsis, skin and kidney disorders, osteoporosis, certain cancers, and hematopoietic dysfunctions without the side effects of known steroid drugs. The diseases listed are usually correlated with aberrant expression of cytokines such as IL-1, TNFα, IL-6, IL-8 that are under the control of NFκB or other rel proteins.

downstream (3' direction) coding sequence. A promoter of a DNA construct, including an oligonucleotide sequence according to the present invention may be linked to a heterologous gene when the presence of the promoter influences transcription from the heterologous gene, including genes for reporter sequences such as luciferase, chloramphenicol acetyl transferase, β-galactosidase and secreted placental alkaline phosphatase.

In a preferred embodiment, the assay is conducted in a whole cell system that has an intracellular receptor which is the target of the screened agent, a promoter or a portion of a promoter with a rel site and a rel-like protein or other transcription protein that binds to the rel site; wherein the intracellular receptor modulates the binding of the rel-like protein or the transcription protein to the rel site. The intracellular receptor, the promoter or a portion of the promoter, or the protein that binds to the rel site may either be endogenous to the cell or transfected into the cell.

In another preferred embodiment, the assay is conducted in an extract of cell having an intracellular receptor, a promoter or a portion of a promoter, with a rel site and a rel-like protein or other protein that binds to the rel site; wherein the intracellular receptor modulates the binding of the rel-like protein or the transcription protein to the rel site.

The binding of the rel-like protein or other transcription protein to the rel site may be measured by techniques known to those skilled in the art, including, but not limited to, mobility shift assay, co-transfection assay, and expression of a reporter gene linked to the promoter.

In a further preferred embodiment, the promoter is activated by an effector, including, but not limited to, tumor necrosis factor, interleukin-1, viruses,

specific treatment of diseases and pathological conditions with little or no effect on healthy tissues.

Other features and advantages of the invention will be apparent from the following detailed description of the invention, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows IL-1 and TNFα induced complex formation on the proximal IL-6 promoter.

Figure 2 shows that several distinct NFκB-related complexes induced by IL-1 and TNFα are modulated by estrogen.

Figure 3 shows the effects of estrogen agonist and antagonist, and inhibitors of protein synthesis and protein kinase C on the formation of NFκB-related complexes.

Figure 4 shows the binding characteristics of proteins in NFκB-related complexes with NFκB oligonucleotides.

Figure 5 shows NFκB related proteins in complexes A, B, and C.

DETAILED DESCRIPTION OF THE INVENTION

A number of cytokines, including IL-6, Il-8 and IL-11, have related biological effects, i.e., effects on cellular defense in response to infection by stimulating the immune and the acute-phase response and on bone metabolism by increasing bone resorption. Aberrant expression of any of these cytokines results in similar pathological conditions, e.g., all cytokines listed are involved in septic shock. In another example, excessive production IL-8, like IL-6, may be involved in the pathogenesis of several types of inflammatory reactions, particularly neutrophil-dependent tissue damages. These cytokines have similar promoter structures, e.g., their promoters contain binding sites for NFκB or other rel

multipotential hemopoietic progenitors," Proc. Natl. Acad. Sci. U.S.A. 84:9035-9039). However, elevated IL-6 expression is usually associated with disease (Yu, X.P., T. Bellido, N. Rice, and S.C. Manolagas (1993).

IL-6 expression is tightly controlled by other factors. Depending on the particular cell type, it can be activated by various stimuli, including tumor necrosis factor (TNFα) and interleukin-1 (IL-1), viruses, endotoxin (lipopolysaccharides), phorbol esters, epidermal growth factor (EGF), leukemia inhibitor factor (LIF), and cAMP agonists.

These effectors exhibit their activity through transcriptional effects on the IL-6 promoter as shown by transfection studies (Gruss, H.J., M.A. Brach, and F. Herrmann (1992) "Involvement of nuclear factor-kappa B in induction of the interleukin-6 gene by leukemia inhibitory factor," Blood 80:2563-2570; Ray, A., S.B. Tatter, L.T. May, and P.B. Sehgal (1988) "Activation of the human "beta 2-interferon/hepatocyte-stimulating factor/interleukin 6" promoter by cytokines, viruses, and second messenger agonists," Proc. Natl. Acad. Sci. U.S.A. 85:6701-6705). By sequence comparison several potential transcriptional control elements have been identified in the IL-6 promoter, including a cAMP response element, an AP-1 binding site, and binding elements for the transcription factors NF-IL6 (C/EBPB, LAP, AGP/EBP) and NFκB (Isshiki, H., S. Akira, O. Tanabe, T. Nakajima, T. Shimamoto, T. Hirano, and T. Kishimoto (1990) "Constitutive and interleukin-1 (IL-1)-inducible factors interact with the IL-1-responsive element in the IL-6 gene," Mol. Cell Biol. 10:2757-2764).

Direct binding of NF-IL6 and NFκB to the IL-6 promoter has been established (Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto (1990) "A

homology to the Drosophila morphogen *dorsal* and to the c-rel proto-oncogeny product. The p65 subunit is also functionally related to c-rel (reviewed in references Baeuerle, P. A. (1991) "The inducible transcription activator NF-kappa B: regulation by distinct protein subunits" Biochim. Biophys. Acta 1072:63-80; Blank, V., P. Kourilsky, and A. Israel (1992) "NF-kappa B and related proteins: Rel/dorsal homologies meet ankyrin-like repeats," Trends. Biochem. Sci. 17:135-140; and Liou, H.C. and D. Baltimore (1993) "Regulation of the NF-kappa B/rel transcription factor and I kappa B inhibitor system," Curr. Opin. Cell Biol. 5:477-487). Recently, additional proteins (p49/p52 and relB/p68) have been identified that are functionally related to p50 and p65 (Henkel, T., T. Machleidt, I. Alkalay, M. Kronke, Y. Ben-Neriah, and P.A. Baeuerle (1993) "Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B," Nature 365:182-185; Perkins, N.D., R.M. Schmid, C.S. Duckett, K. Leung, N.R. Rice, and G.J. Nabel (1992) "Distinct combinations of NF-kappa B subunits determine the specificity of transcriptional activation," Proc. Natl. Acad. Sci. U.S.A. 89:1529-1533; Ryseck, R.P., P. Bull, M. Takamiya, V. Bours, U. Siebenlist, P. Dobrzanski, and R. Bravo (1992) "RelB, a new Rel family transcription activator that can interact with p50-NF-kappa B," Mol. Cell Biol. 12:674-684; Ryseck, R.P., P. Bull, M. Takamiya, V. Bours, U. Siebenlist, P. Dobrzanski, and R. Bravo (1992) "RelB, a new Rel family transcription activator that can interact with p50-NF-kappa B," Mol. Cell Biol. 12:674-684; Schmid, R.M., N.D. Perkins, C.S. Duckett, P.C. Andrews, and G.J. Nabel (1991) "Cloning of an NF-kappa B subunit which stimulates HIV transcription in synergy with p65," Nature 352:733-736). NFκB is located in the cytosol complexes with an inhibitory protein of the IκB family. Upon induction, NFκB

osteoclastogenesis compared with cultures from sham-operated animals. This increase in osteoclast development can be prevented by injection of an anti-IL-6 antibody or by administration of estrogen (Jilka, R.L., G. Hangoc, G. Girasole, G. Passeri, D.C. Williams, J.S. Abrams, B. Boyce, H. Broxmeyer, and S.C. Manolagas (1992) "Increased osteoclast development after estrogen loss: mediation by interleukin-6," Science 257:88-91). In mice that carry a null mutation for IL-6, ovariectomy does not affect bone volume or osteoclast number as seen with normal mice (Balena, R., F. Costantini, M. Yamamoto, A. Markatos, R. Cortese, G.A. Rodan, and V. Poli (1993) "Mice with IL-6 gene knock-out do not lose cancellous bone after ovariectomy," J. Bone Miner. Res. 8:S130 [Abstract]).

Regulation of Interleukin 6 by Estrogen

Estrogen has been found to inhibit IL-6 expression in bone-derived stromal cell lines and osteoblastic cells from rats and mice as well as in nontransformed human bone cells (Girasole, G., R.L. Jilka, G. Passeri, S. Boswell, G. Boder, D.C. Williams, and S.C. Manolagas (1992) "17 beta-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in vitro: a potential mechanism for the antiosteoporotic effect of estrogens," J. Clin. Invest. 89:883-891). This effect of estrogen on IL-6 expression is not restricted to bone tissue but has also been shown for uterine cells (Jacobs, A.L., P.B. Sehgal, J. Julian, and D.D. Carson (1992) "Secretion and hormonal regulation of interleukin-6 production by mouse uterine stromal and polarized epithelial cells cultured in vitro," Endocrinology 131:1037-1046; Tabibzadeh, S.S., U. Santhanam, P.B. Sehgal, and L.T. May (1989) "Cytokine-induced production of IFN-beta 2/IL-6 by freshly explanted human endometrial stromal cells. Modulation by estradiol-17 beta," J. Immunol. 142:3134-

proteins. Only anti-p50 and anti-p65 had an effect and abolished the formation of the induced complex.

An oligonucleotide covering the potential NFκB site of the IL-6 promoter competed against the induced binding to this fragment, while an oligonucleotide covering the NF-IL6 site was ineffective. When the NFκB oligonucleotide was used as probe, three IL-1/TNFα-induced complexes were observed.

Pretreatment with estradiol decreased the intensity of the slowest complex and strongly increased the intensity of the fastest migrating complex. The three bands were differentially supershifted (i.e., further decrease in the mobility of the complex due to binding of the antibody) by anti-p50 and anti-p65 antibodies, while none of several other antibodies tested, including anti-ER antibody, had any effect. Methylation interference assays showed identical DNA contact sites for all three complexes.

Ray, et al., J. Biol. Chem., 269(17):12940-946 (1994), not admitted to be prior art, describe that activation of the IL-6 promoter, elicited by a combination of NF-IL6 and the p65 subunit of NFκB, can be inhibited by the wt ER but not by an ER containing a mutation in its DNA binding domain. Furthermore, the repression of the IL-6 promoter by a combination ER and 17β-estradiol did not appear to be mediated via high affinity binding of the receptor to the promoter.

These data suggest that negative regulation by estrogen is mediated through the IL-6 promoter and is estrogen receptor dependent. Inhibition of IL-6 expression by estrogen is mediated through control of the transcriptional activity of NFκB or closely related proteins on the IL-6 promoter.

Mukaida, et al., J. Biol. Chem., 269(18):13289-295 (1994), not admitted to be prior art, describe that a glucocorticoid, dexamethasone, inhibited IL-8 production

measuring of cytokine using standard assays known to those skilled in the art.

2) Measuring the expression of a reporter introduced into the cell.

By means of transfection a reporter construct will be introduced into the cells that expresses an easily measurable protein under the control of a cytokine promoter or fragments thereof or isolated rel-sites. The other necessary components are either expressed endogenously by the cells or provided by cotransfection of expression vectors for the particular component. Cells will be treated with the agent or vehicle control and an effector (phorbol ester, cytokines, lipopolysaccharides). The activity of the agent will be analyzed quantitatively by measuring the expression of the reporter protein.

Agents will also be tested for their binding to IRs by traditional binding assays as well as for their activity to effect the classical mechanism of gene regulation by IRs. An agent that binds to IRs and regulates binding of rel proteins to cytokine promoters but does not activate the classical mechanism of IR action is a potential drug candidate for the specific treatment of diseases associated with aberrant expression of cytokines.

Experimental procedures employed in the examples described herein are set forth below:

Transient transfections and mammalian expression constructs

Construction of the pERE-tk-Luc reporter plasmid and the vector expressing ER_{gly} (pRShER) has been described (Tzukerman, M., A. Esty, D. Santiso-Mere, P. Danielian, M.G. Parker, R.B. Stein, J.W. Pike, and D.P. McDonnell (1994) "Human estrogen receptor transactivational capacity is determined by both cellular and promoter

Antibodies, IL-6 ELISA, and ER assay

Peptides used to raise the following antibodies in rabbits correspond to amino acid residues 91-105 of murine c-jun (Ryder and Nathans (1988) "Induction of protooncogene c-jun by serum growth factors," Proc. Natl. Acad. Sci. USA 85:8464-8467), 278-296 of murine NF-IL6 (Chang, C.J., T.T. Chen, H.Y. Lei, D.S. Chen, and S.C. Lee (1990) "Molecular cloning of a transcription factor, AGP/EBP, that belongs to members of the C/EBP family," Mol. Cell Biol. 10:6642-6653), 152-176 of murine c-rel (Bull, P., K.L. Morley, M.F. Hoekstra, T. Hunter, and I.M. Verma (1990) "The mouse c-rel protein has an N-terminal regulatory domain and a C-terminal transcriptional transactivation domain," Mol. Cell Biol. 10:5473-5485; Inoue, J., L.D. Kerr, L.J. Ransone, E. Bengal, T. Hunter, and I.M. Verma (1991) "c-rel activates but v-rel suppresses transcription from kappa B sites," Proc. Natl. Acad. Sci. U.S.A. 88:3715-3719), 347-361 of murine p50 (Ghosh, S., A.M. Gifford, L.R. Riviere, P. Tempst, G.P. Nolan, and D. Baltimore (1990) "Cloning of the p50 DNA binding subunit of NF-kappa B: homology to rel and dorsal," Cell 62:1019-1029), and 3-19 of human p65 (88% homology with murine p65) (Nolan, G.P., S. Ghosh, H.C. Liou, P. Tempst, and D. Baltimore (1991) "DNA binding and I kappa B inhibition of the cloned p65 subunit of NF-kappa B, a rel-related polypeptide," Cell 64:961-969; Ruben, S.M., P.J. Dillon, R. Schreck, T. Henkel, C.H. Chen, M. Maher, P.A. Baeuerle, and C.A. Rosen (1991) "Isolation of a rel-related human cDNA that potentially encodes the 65-kD subunit of NF-kappa B [letter]," Science 254:11). All the references mentioned above are incorporated by reference herein. All antibodies listed above were obtained affinity purified at a concentration of 1 mg/ml from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-TBP was a protein-A purified serum preparation from

J. Clin. Invest. 89:883-891). To prepare nuclear extracts the cells were seeded in phenol-red-free McCoy's medium supplemented with 10% FBS and pretreated with hormone for 24 h if not indicated otherwise. After adjusting the medium to 2% FBS, the cells were induced with TNFα and IL-1b (1 nM each) for varying periods. In cases where cycloheximide (10 mg/ml) or the kinase inhibitor H7 (50 mM) were included, those compounds were added 5 min before induction. Incubation was stopped by two washes with ice cold PBS and cells were lysed in situ in cold buffer A (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2% Nonidet P-40). Lysates were transferred into microfuge tubes, nuclei pelleted (8000 rpm, 1 min) and resuspended in buffer C (20 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 420 mM NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF). After 40 min rocking at 4 °C, samples were centrifuged (15,000 rpm, 10 min) and supernatants taken as nuclear extracts. Bradford protein assays (Bradford, M.M. (1976) "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," Anal. Biochem. 72:248-254) showed only minimal variations in protein concentrations which did not correlate with hormone or cytokine treatment. Extracts of yeast recombinantly expressing ER_{gly} were prepared from the BJ2168 strain transformed with YEpE10 as described (Tzukerman, M., A. Esty, D. Santiso-Mere, P. Danielian, M.G. Parker, R.B. Stein, J.W. Pike, and D.P. McDonnell (1994) "Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions," Mol. Endocrinol. 8:21-30). Purified, *Escherichia coli* expressed human p50 and p49 proteins were purchased from Promega (Madison, WI).

LeBowitz, A.S. Baldwin, Jr., and P.A. Sharp (1988) "Molecular cloning of an enhancer binding protein: isolation by screening of an expression library with a recognition site DNA," Cell 52:415-423). After autoradiography, the DNA corresponding to the various complexes and the unretarded probe was eluted (10 min at 65 °C in 20 mM Tris [pH 8.0], 1 M NaCl, 0.1 mM EDTA) and purified by phenol/chloroform extraction and ethanol precipitation. After strand cleavage in 1 M piperidine (30 min at 90 °C) the fragments were resolved on denaturing polyacrylamide gels (12% acrylamide/0.6% BIS).

Example 1. Screening for ER mediated inhibition of IL-6 promoter activity

It has been shown that IL-6 repression is regulated by estradiol at the mRNA level (Girasole, G., R.L. Jilka, G. Passeri, S. Boswell, G. Boder, D.C. Williams, and S.C. Manolagas (1992) "17 beta-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in vitro: a potential mechanism for the antiosteoporotic effect of estrogens," J. Clin. Invest. 89:883-891; Jacobs, A.L., P.B. Sehgal, J. Julian, and D.D. Carson (1992) "Secretion and hormonal regulation of interleukin-6 production by mouse uterine stromal and polarized epithelial cells cultured in vitro," Endocrinology 131:1037-1046). To determine if estrogen or a candidate agent acts directly on IL-6 transcription, we transfected a reporter construct, expressing the firefly luciferase under the control of the human IL-6 promoter region from -225 to +14, into the murine fibroblast cell line C3H10T1/2. These cells can be considered as pre-osteoblasts since they differentiate into osteogenic cells in response to bone morphogenic protein-2 (Katagiri, T., A. Yamaguchi, T. Ikeda, S. Yoshiki, J.M. Wozney, V. Rosen, E.A. Wang, H.

activator and repressor functions in the absence of ligand," New Biol. 2:613-620).

The dependence of the estrogen effect on cotransfected ER suggested that C3H10T1/2 cells do not express functional endogenous ER. This was confirmed by transfecting the cell with a luciferase reporter under the control of the vitellogenin estrogen response element (ERE) (Klein-Hitpass, L., M. Schorpp, U. Wagner, and G.U. Ryffel (1986) "An estrogen-responsive element derived from the 5' flanking region of the Xenopus vitellogenin A2 gene functions in transfected human cells," Cell 46:1053-1061).

Therefore, C3H10T1/2 cells were transfected with a luciferase expression vector under the control of the minimal thymidine kinase promoter and the vitellogenin estrogen response element (pERE-tk-Luc) alone or together with pRShER. 24 h after treatment with 10 nM estradiol or vehicle cells were harvested and extracts analyzed for luciferase activity. Induction of luciferase activity by estradiol was only observed in the presence of cotransfected ER.

In addition, C3H10T1/2 cells were incubated with or without 10 nM estradiol. After 24 h the cultures were induced with TNFα and IL-1 (1 nM each) or left uninduced for additional 24 h. Il-6 in the supernatants was assayed by an ELISA specific for murine IL-6. C3H10T1/2 cells responded to IL-1 and TNFα treatment with strongly increased production of endogenous IL-6, but unlike other osteogenic or stromal cells containing endogenous ER (Girasole, G., R.L. Jilka, G. Passeri, S. Boswell, G. Boder, D.C. Williams, and S.C. Manolagas (1992) "17 beta-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in vitro: a potential mechanism for the antiosteoporotic effect of estrogens," J. Clin. Invest. 89:883-891), IL-6 levels were not decreased by estradiol. These data suggest

secretion of IL-6. Treatment with estradiol inhibits this induction of IL-6 as shown for the protein and its mRNA (Girasole, G., R.L. Jilka, G. Passeri, S. Boswell, G. Boder, D.C. Williams, and S.C. Manolagas (1992) "17 beta-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in vitro: a potential mechanism for the antiosteoporotic effect of estrogens," J. Clin. Invest. 89:883-891).

To verify that ER is actually present in +/+LDA11 cells, hormone binding studies were carried out. Initial experiments showed a low number of specific estradiol binding sites in high salt extracts from these cells. Using a monoclonal antibody directed against the amino terminus of the ER, specifically bound [^{3}H]estradiol was immunoprecipitated confirming that the binding sites represented ER. From those studies we calculated that +/+LDA11 cells contain approximately 1000 ER molecules per cell.

However, when using electrophoretic mobility shift assays (EMSA) in combination with the vitellogenin ERE as a probe, ER-specific DNA binding activity could not be detected in nuclear extracts from +/+LDA11 treated with estradiol and/or IL-1 and TNFα. Nuclear extracts of +/+LDA11 cells pretreated with estradiol (10 nM) and TNFα and IL-1 (1nM each for 40 min) as indicated or yeast extract containing recombinantly expressed human wild-type ER_{gly} were incubated with the vitellogenin ERE as probe in the absence or presence of anti-ER antibody. Complexes formed were analyzed by EMSA.

The complexes detected are unrelated to the ER since they were not significantly affected by anti-ER antibody. Controls using ER containing extracts obtained from a yeast expression system gave rise to two slowly migrating complexes that were specifically shifted with the anti-ER antibody. These data suggest

After 24 h the cells were induced with TNFα and IL-1 (1 nM each) for various periods of time. Induction was stopped by cell lysis and nuclear extracts were analyzed by EMSA using the -225 to -52 IL-6 promoter fragment as probe (Figure 1a).

Complexes formed were analyzed by EMSA. After treatment with the cytokines an inducible complex was observed. The intensity of the complex was maximal already after 10 min treatment with IL-1 and TNFα and decreased gradually over time. After 2 hours of induction the intensity of the complex was significantly reduced.

Pretreatment of the cells with estradiol had no effect on the binding capacity of extracts from uninduced cells. However, estradiol pretreatment resulted in a marked increase of the induced complex with induction intervals from 10 min to 40 min but only a slight effect on the complex after 2 h of induction. In addition to the increased intensity, pretreatment with estradiol also caused a qualitative change, increasing the mobility of the complex.

Detecting the composition of the DNA-binding complex

To investigate the nature of the complex and the proteins potentially involved, we incubated the binding reactions with antibodies directed against several potential binding factors. Nuclear extract from +/+LDA11 cells treated with estradiol (10 nM) and TNFα and IL-1 (1nM each for 10 min) as indicated were incubated with the -225 to -52 probe in the absence or presence of various antibodies. Complexes formed were analyzed by EMSA.

Fig. 1b shows that none of the antibodies tested affected DNA binding of extracts from uninduced cells. Neither anti-c-jun, nor anti-c-rel, nor anti-NF-IL6

probe in the absence or presence of a 400-fold molar excess of oligonucleotides corresponding to the regions of -82 to -47 and -172 to -131 of the human IL-6 promoter. Complexes formed were analyzed by EMSA.

The arrows in Fig. 1d indicate the complexes formed upon induction with TNFα and IL-1. Inclusion of an oligonucleotide covering the NF-IL6 site, the CRE, and an adjacent CCAAT-box of the IL-6 promoter (-172 to -131) in the binding reaction in 400-fold excess over the labeled -225 to -52 fragment did not affect any of the complexes, constitutive or cytokine-induced (lanes 4 and 7). However, an oligonucleotide covering the putative NFκB site and adjacent sequences (-82 to -47) specifically abolished the formation of the cytokine induced complexes (lanes 3 and 6).

The antibody experiments and the oligonucleotide competition studies suggested that IL-1 and TNFα specifically activated NFκB or related proteins. No binding of c-jun (AP-1), NF-IL6, c-rel, or ER was detected.

The lack of NF-IL6 binding is surprising, since induction and binding of this transcription factor in response to IL-1 has been reported for other cells (Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto (1990) "A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family," EMBO J. 9:1897-1906; Inoue, J., L.D. Kerr, L.J. Ransone, E. Bengal, T. Hunter, and I.M. Verma (1991) "c-rel activates but v-rel suppresses transcription from kappa B sites," Proc. Natl. Acad. Sci. USA 88:3715-3719). Our DNA binding experiments show that in the bone marrow derived +/+LDA11 cell IL-1 and TNFα induce the binding of NFκB or closely related proteins to the IL-6 promoter. Similar results have been obtained in different cell types (H. Shimizu, K. Mitomo, T. Watanabe, S. Okamoto,

activities," Mol. Cell Biol. 11:6016-6025). Several reports also suggest a cross-talk between ER and AP-1, however there is no evidence for estrogen dependent inhibition of AP-1 activity (Gaub, M.P., M. Bellard, I. Scheuer, P. Chambon, and P. Sassone-Corsi (1990) "Activation of the ovalbumin gene by the estrogen receptor involves the fos-jun complex," Cell 63:1267-1276; Tzukerman, M., X.K. Zhang, and M. Pfahl (1991) "Inhibition of estrogen receptor activity by the tumor promoter 12-O-tetradeconylphorbol-13-acetate: a molecular analysis," Mol. Endocrinol. 5:1983-1992). Taken together, it is highly unlikely that AP-1 plays a role in the negative regulation of IL-6 expression by estrogen.

Example 3. Screening agents that differentially affect distinct complexes with the IL-6 promoter

Distinctive complexes with the IL-6 promoter

Treatment of +/+LDA11 cells with IL-1 and TNFα induced binding of NFκB or related proteins to the IL-6 promoter. Since pretreatment with estradiol not only increased the intensity but also the mobility of the complexes, we investigated the binding of +/+LDA11 nuclear extracts to the oligonucleotide covering the putative NFκB site (-82 to -47).

+/+LDA11 cells were pretreated with 10 nM estradiol as indicated. After 24 h the cells were induced with TNFα and IL-1 (1 nM each) for various periods of time. Induction was stopped by cell lysis and nuclear extracts were analyzed by EMSA using the -82 to -47 IL-6 promoter fragment as probe. Fig. 2a shows that extracts from cells treated with IL-1 and TNFα exhibited 3 induced complexes (A,B,C) when compared with extracts from untreated cells.

the complexes A, B, and C formed upon induction with TNFα and IL-1.

Two of the complexes were specific, since they could be competed with an excess of the unlabeled oligonucleotide (lanes 2, 4, and 6). However, all of the complexes were formed constitutively, independent of cytokine induction or estradiol treatment, suggesting that they were unrelated to the regulation of IL-6 expression by IL-1, TNFα, and estrogen.

Screening for compounds that affect the formation of distinct complexes

In more detailed studies we analyzed the effects of other compounds on the formation of complexes A, B, and C (Fig. 3). +/+LDA11 cells were pretreated with cycloheximide (CHX) or the kinase inhibitor H7 for 5 min or with estradiol (10 nM) and/or ICI 164,384 (100 nM) for 24 h or 60 min before induction with TNFα and IL-1 (1nM each for 30 min). Treatment was stopped by cell lysis and nuclear extracts were analyzed by EMSA using the -82 to -47 fragment as probe. Arrows indicate the induced complexes A, B, and C.

Pretreatment of the cells with the protein synthesis inhibitor cycloheximide before addition of cytokines did not interfere with complex formation (lane 8). This is consistent with the fast induction of binding and has been shown before for the activation of NFκB (Henkel, T., T. Machleidt, I. Alkalay, M. Kronke, Y. Ben-Neriah, and P.A. Baeuerle (1993) "Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B," Nature 365:182-185; Sen, R. and D. Baltimore (1986) "Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism," Cell 47:921-928). It has been reported that cycloheximide treatment activates NFκB binding (Sen, R. and D. Baltimore (1986)

164,384 was added in combination with estradiol it prevented the effect mediated by the estrogen (lane 5).

Since ICI 164,384 acts as an antagonist via binding to the ER, these results further support the hypothesis that the effects of estradiol on the induced complexes are receptor mediated. However, the mechanism of estrogen action is probably indirect, as indicated by the lack of response to short term estradiol treatment.

Screening for agents that affect binding characteristics of the proteins in distinct complexes

To investigate the binding characteristics of the proteins in complexes A, B, and C with the NFκB oligonucleotide, methylation interference experiments were carried out (Fig. 4). Nuclear +/+LDA11 extracts from cells induced with TNFα and IL-1 (1nM each) were incubated with -82 to -47 probe that had been labeled either on the upper or the lower strand and subjected to limited DMS-methylation. After preparative EMSA, DNA from complexes A, B, and C and from the unretarded probe (F) was isolated, cleaved with piperidine, and electrophoresed on a 12% denaturing gel. The sequence corresponding to the NFκB consensus site is shown boxed, a cryptic AP-1 site is shaded.

On both strands N-7-methylation of the guanine bases within the NFκB site (-73 to -63, boxed) interfered with complex formation, while methylation of guanines flanking the consensus site had no observable effect. The interference pattern for all three complexes (A,B,C) was identical.

The observation that methylation of guanines just outside of the NFκB site (-75, -60, -58) did not affect the formation of even the largest complex (A) suggests that in all three complexes DNA contacts are made within the same core region. In addition, the lack of DNA binding interference with methylation of guanines -60, -

10:1817-1825). C-rel homodimers and heterodimers with p50 have been shown to bind the NFκB site in the IL-6 promoter (Nakayama, K., H. Shimizu, K. Mitomo, T. Watanabe, S. Okamoto, and K. Yamamoto (1992) "A lymphoid cell-specific nuclear factor containing c-Rel-like proteins preferentially interacts with interleukin-6 kappa B-related motifs whose activities are repressed in lymphoid cells," Mol. Cell Biol. 12:1736-1746).

In addition, this study showed that in lymphoid cells c-rel or an immunologically related factor is a component of a larger complex that binds the NFκB site in the IL-6 promoter and functions as a constitutive repressor. In +/+LDA11 cells, we could not detect any c-rel specific binding activity. A number of other NFκB unrelated proteins have been shown to bind to NFκB consensus sites. Those include aA-CRYBP1 (Nakamura, T., D.M. Donovan, K. Hamada, C.M. Sax, B. Norman, J.R. Flanagan, K. Ozato, H. Westphal, and J. Piatigorsky (1990) "Regulation of the mouse alpha A-crystallin gene: isolation of a cDNA encoding a protein that binds to a cis sequence motif shared with the major histocompatibility complex class I gene and other genes," Mol. Cell Biol. 10:3700-3708), MBP-1/PRDII-BFI (Baldwin, A.S., Jr., K.P. LeClair, H. Singh, and P.A. Sharp (1990) "A large protein containing zinc finger domains binds to related sequence elements in the enhancers of the class I major histocompatibility complex and kappa immunoglobulin genes," Mol. Cell Biol. 10:1406-1414; Fan, C.M. and T. Maniatis (1990) "A DNA-binding protein containing two widely separated zinc finger motifs that recognize the same DNA sequence," Genes Dev. 4:29-42), and AGIE-BP1 (Ron, D., A.R. Brasier, and J.F. Habener (1991) "Angiotensinogen gene-inducible enhancer-binding protein 1, a member of a new family of large nuclear proteins that recognize nuclear

the binding reactions. Interestingly, anti-p50 specifically abolished the formation of complexes B and C, seemed to leave complex A unaffected, and caused the appearance of a single supershifted band (S1). Anti-p65, however, inhibited the formation of all three induced complexes and produced two supershifted bands (S1, S2). This suggested that p65 or an immunologically closely related protein is part of all three induced complexes, while p50 or a related protein is only present in complexes B and C.

It has been reported that recombinantly expressed c-rel binds to the NFκB site in the IL-6 promoter as heterodimer with p50 and, with particular high affinity, as homodimer (Nakayama, K., H. Shimizu, K. Mitomo, T. Watanabe, S. Okamoto, and K. Yamamoto (1992) "A lymphoid cell-specific nuclear factor containing c-Rel-like proteins preferentially interacts with interleukin-6 kappa B-related motifs whose activities are repressed in lymphoid cells," Mol. Cell Biol. 12:1736-1746). When anti-c-rel was included in the binding reactions with the +/+LDA11 extracts the antibody did not inhibit any of the induced complexes. On longer exposures a weak supershifted complex was detectable. This complex migrated at the same position as the supershift observed with anti-p50 suggesting that it did not contain the larger c-rel protein. Since the peptide used to raise the anti-c-rel antibody has a 56% homology to the analogous p50 sequence (Ghosh, S., A.M. Gifford, L.R. Riviere, P. Tempst, G.P. Nolan, and D. Baltimore (1990) "Cloning of the p50 DNA binding subunit of NF-kappa B: homology to rel and dorsal," Cell 62:1019-1029; Inoue, J., L.D. Kerr, L.J. Ransone, E. Bengal, T. Hunter, and I.M. Verma (1991) "c-rel activates but v-rel suppresses transcription from kappa B sites," Proc. Natl. Acad. Sci. USA 88:3715-3719), it is likely that this weak band is the result of a cross-reactivity and unrelated to c-

affect the migration, suggesting that the band represented p50 homodimers and not higher order complexes (Duckett, C.S., N.D. Perkins, T.F. Kowalik, R.M. Schmid, E.S. Huang, A.S. Baldwin, Jr., and G.J. Nabel (1993) "Dimerization of NF-KB2 with RelA(p65) regulates DNA binding, transcriptional activation, and inhibition by an I kappa B-alpha (MAD-3)," Mol. Cell Biol. 13:1315-1322). However, the antibody shift experiments suggested that both NFκB proteins, p50 and p65, are part of complexes B and C (lanes 6, 7, 11, and 12) and consequently both complexes should migrate slower than p50 homodimers (Urban, M.B., R. Schreck, and P.A. Baeuerle (1991) "NF-kappa B contacts DNA by a heterodimer of the p50 and p65 subunit," EMBO J. 10:1817-1825).

It is possible that the proteins in complexes B and C are only immunologically related to p50 and p65 but actually of smaller size. Speculations that the p50 homologue p49 is part of the induced complexes and is responsible for the faster migration could not be confirmed. Although purified p49 bound the -82 to -47 IL-6 fragment and strongly cross-reacted with the anti-p50 antibody, the complex formed migrated even more slowly than the p50 complex. This corresponds to results obtained with other NFκB binding sites (Duckett, C.S., N.D. Perkins, T.F. Kowalik, R.M. Schmid, E.S. Huang, A.S. Baldwin, Jr., and G.J. Nabel (1993) "Dimerization of NF-KB2 with RelA(p65) regulates DNA binding, transcriptional activation, and inhibition by an I kappa B-alpha (MAD-3)," Mol. Cell Biol. 13:1315-1322).

However, the finding that the anti-p50 antibody strongly cross-reacts with p49 indicates that the antibodies used may detect other NFκB related proteins in the complexes. Alternatively, the migration of complexes B and C could be higher than the migration

induced complexes. However, using an anti-TBP antibody we could not detect any participation of TBP in complexes A, B, or C.

Our antibody gel shift experiments suggested that p65 is a component of all three observed complexes. This particular protein is the NFκB component containing the transactivation domain (Schmitz, M.L. and P.A. Baeuerle (1991) "The p65 subunit is responsible for the strong transcription activating potential of NF-kappa B," EMBO J. 10:3805-3817). Within the different complexes the transactivation function may be differentially active. The antibody shift experiments suggest that estradiol diminishes the A2 complex while increasing complex C. The slow migrating A2 complex may contain other factor(s) involved in the transactivation process. The TATA-binding protein TBP, part of the TFIID complex has been reported to interact strongly with c-rel and p65, but not with p50 or p49 (Kerr, L.D., L.J. Ransone, P. Wamsley, M.J. Schmitt, T.G. Boyer, Q. Zhou, A.J. Berk, and I.M. Verma (1993) "Association between proto-oncoprotein Rel and TATA-binding protein mediates transcriptional activation by NF-kappa B," Nature 365:412-419). However, using a TBP-specific antibody we could not detect TBP as part of any of the complexes formed with the NFκB site in the IL-6 promoter.

Example 5. Efficacy-testing of Putative Cytokine Modulators

Methods for testing the efficacy of putative cytokine modulators are provided. Each candidate compound is tested for its efficacy in modulating cytokine expression in cell lines, in animal models, and in controlled clinical studies using methods known to those skilled in the art and approved by the Food and Drug Administration, such as, but not limited to, those

in the tetrazole ring of XTT to form XTT formazan. Dead cells or cells with impaired energy metabolism are incapable of this cleavage reaction. The extent of the cleavage is directly proportional to the number of living cells tested. Cells from a human cell line such as HeLa cells are seeded at 10^3 per well in 0.1 ml of cell culture medium (Dulbecco' s modified minimal essential medium supplemented with 10% fetal calf serum) in the wells of a 96 well microtiter plate. Cells are allowed to adhere to the plate by culture at 37° C in an atmosphere of 95% air, 5% CO_2. After overnight culture, solutions of test substances are added in duplicate to wells at concentrations that represent eight half-decade log dilutions. In parallel, the solvent used to dissolve the test substance is added in duplicate to other wells. The culture of the cells is continued for a period of time, typically 24 hours. At the end of that time, a solution of XTT and a coupler (methylphenazonium sulfate) is added to each of the test wells and the incubation is continued for an additional 4 hours before the optical density in each of the wells is determined at 450 nm in an automated plate reader. Substances that kill mammalian cells, or impair their energy metabolism, or slow their growth are detected by a reduction in the optical density at 450 nm in a well as compared to a well which received no test substance.

B. Additional screens for Toxicity: Method 2

Cytokine modulators are tested for cytotoxic effects on cultured human cell lines using incorporation of ^{35}S methionine into protein as an indicator of cell viability. HeLa cells are grown in 96 well plates in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum and 50μg/ml penicillin and streptomycin. Cells are initially seeded at 10^3 cells/well, 0.1 ml/well. Cells are grown for 48 hrs without exposure to the cytokine modulator, then medium

Furthermore, the invention features a method for treating a subject inflicted with a pathological condition affected by the level of a cytokine by administering to that subject a therapeutically effective amount of a cytokine modulator. Such administration can be by any method known to those skilled in the art, for example, by topical application or by systemic administration.

By "therapeutically effective amount" is meant an amount that relieves (to some extent) one or more symptoms of the disease or condition in the patient. Additionally, by "therapeutically effective amount" is meant an amount that returns to normal, either partially or completely, physiological or biochemical parameters associated with or causative of a mycotic disease or condition. Generally, it is an amount between about 1 nmole and 1 μmole of the molecule, dependent on its EC_{50} and on the age, size, and disease associated with the patient.

Other embodiments of this invention are disclosed in the following claims.

9. The method of claim 1, wherein said condition is Kaposi's sarcoma.

10. The method of claim 1, wherein said condition is septic shock.

11. The method of claim 1, wherein said condition is multiple myeloma.

12. The method of claim 1, wherein said intracellular receptor is a steroid receptor.

13. The method of claim 1, wherein said intracellular receptor is an estrogen receptor.

14. The method of claim 1, wherein said intracellular receptor is selected from the group consisting of retinoid acid receptors, retinoid X receptors, glucocorticoid receptor, progesterone receptors, androgen receptor, thyroid hormone receptors, and vitamin D receptor.

15. The method of claim 1, wherein said measuring comprises determining the expression level of a cytokine or an acute phase protein.

16. The method of claim 1, wherein said measuring comprises determining the expression level of a reporter gene linked to said promoter.

17. The method of claim 1, wherein said system further comprises an effector of said promoter.

18. The method of claim 17, wherein said effector is selected from the group consisting of tumor necrosis factor, interleukin-1, viruses, endotoxin, phorbol

FIG. 1a.

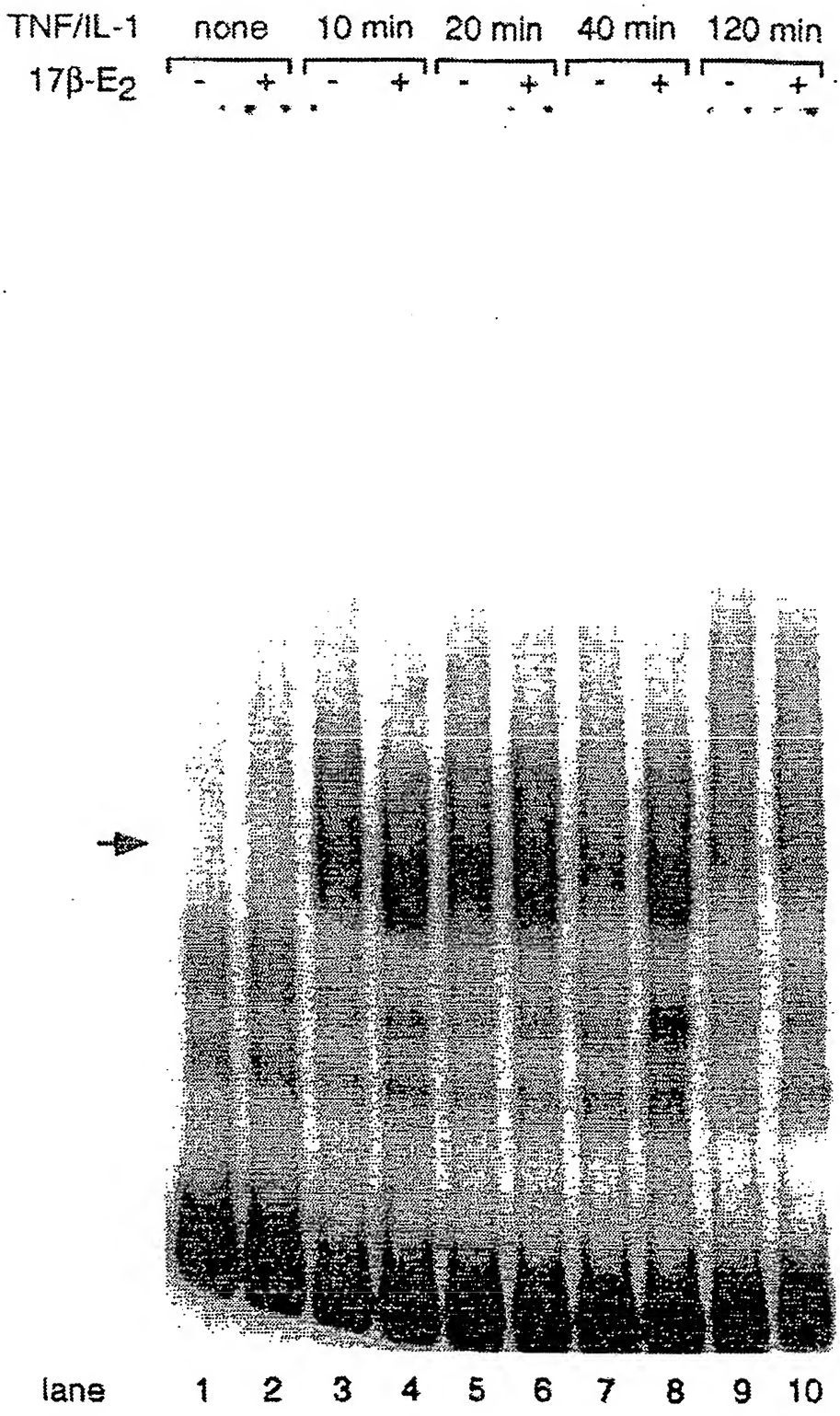


FIG. 1c.

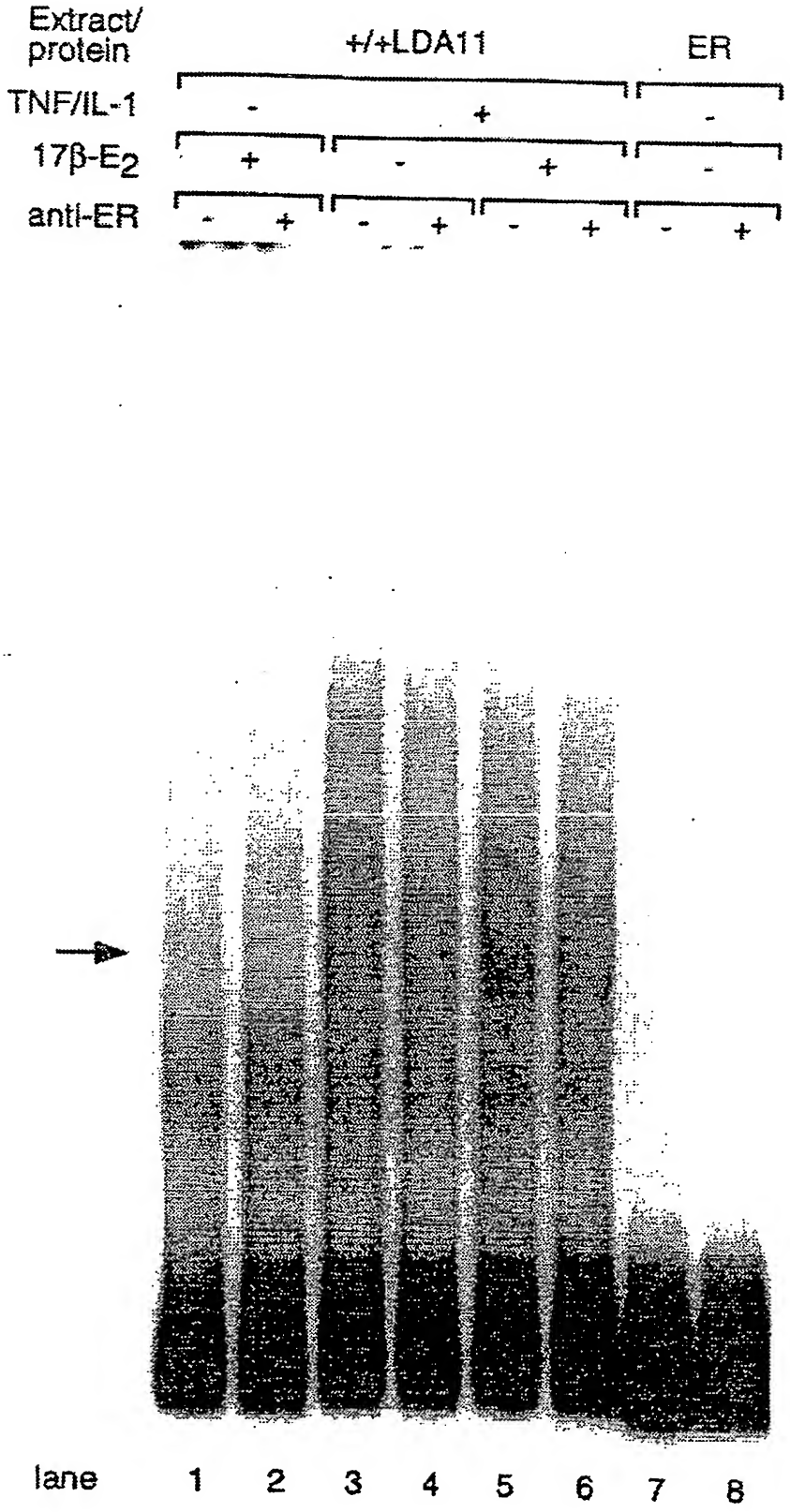
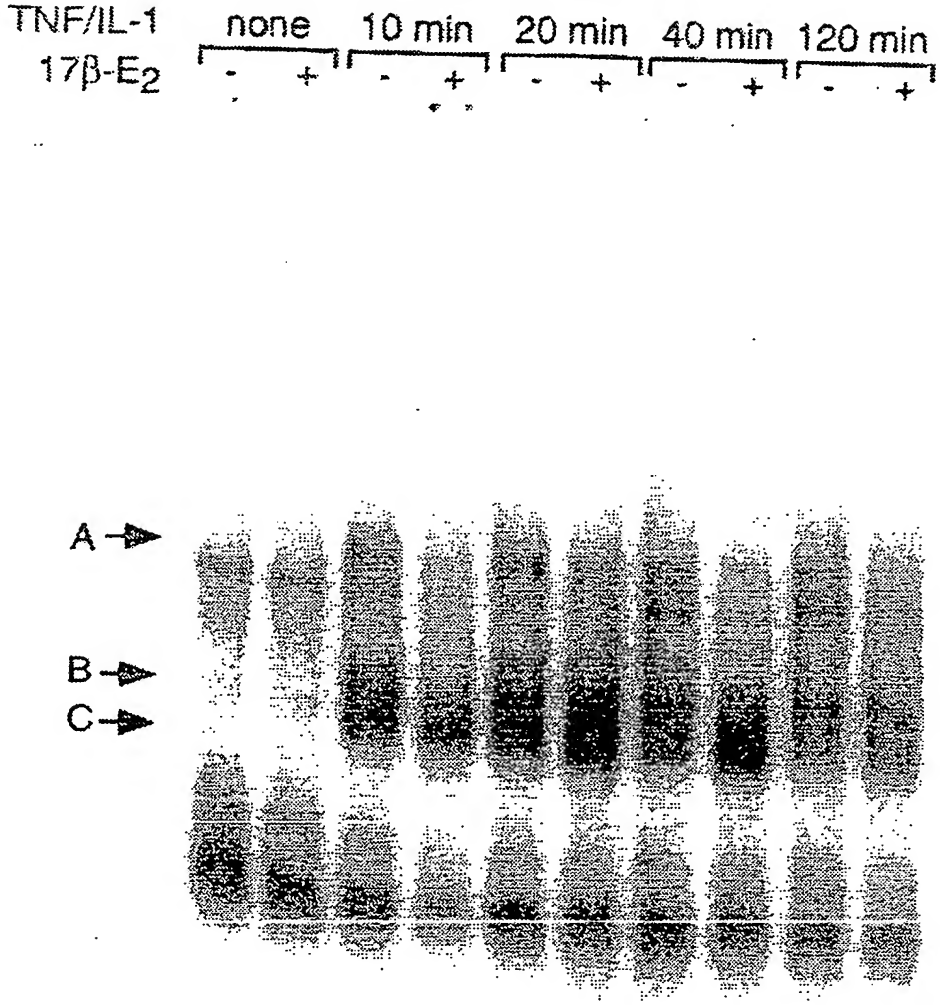


FIG. 2a.



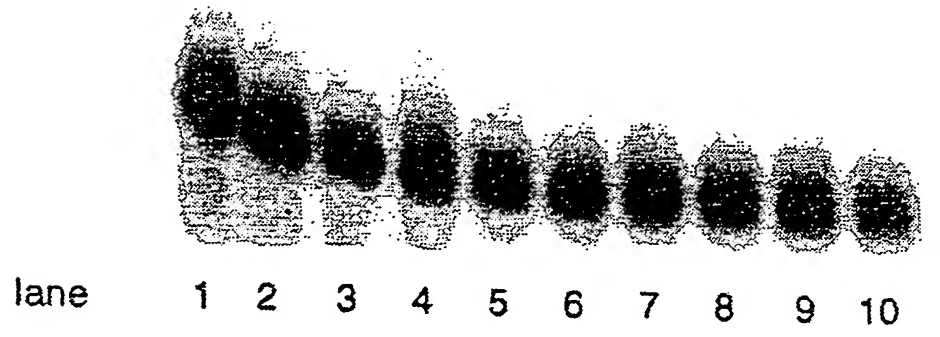
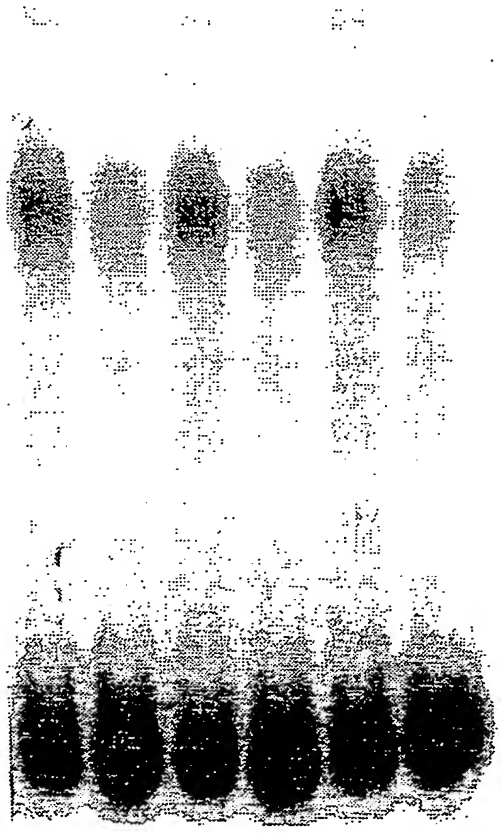


FIG. 2c.

1/11

Induction	none		TNF/IL-1			
17β-E_2	+		-		+	
Competitor	none	172-131	none	172-131	none	172-131



lane 1 2 3 4 5 6

FIG. 4.

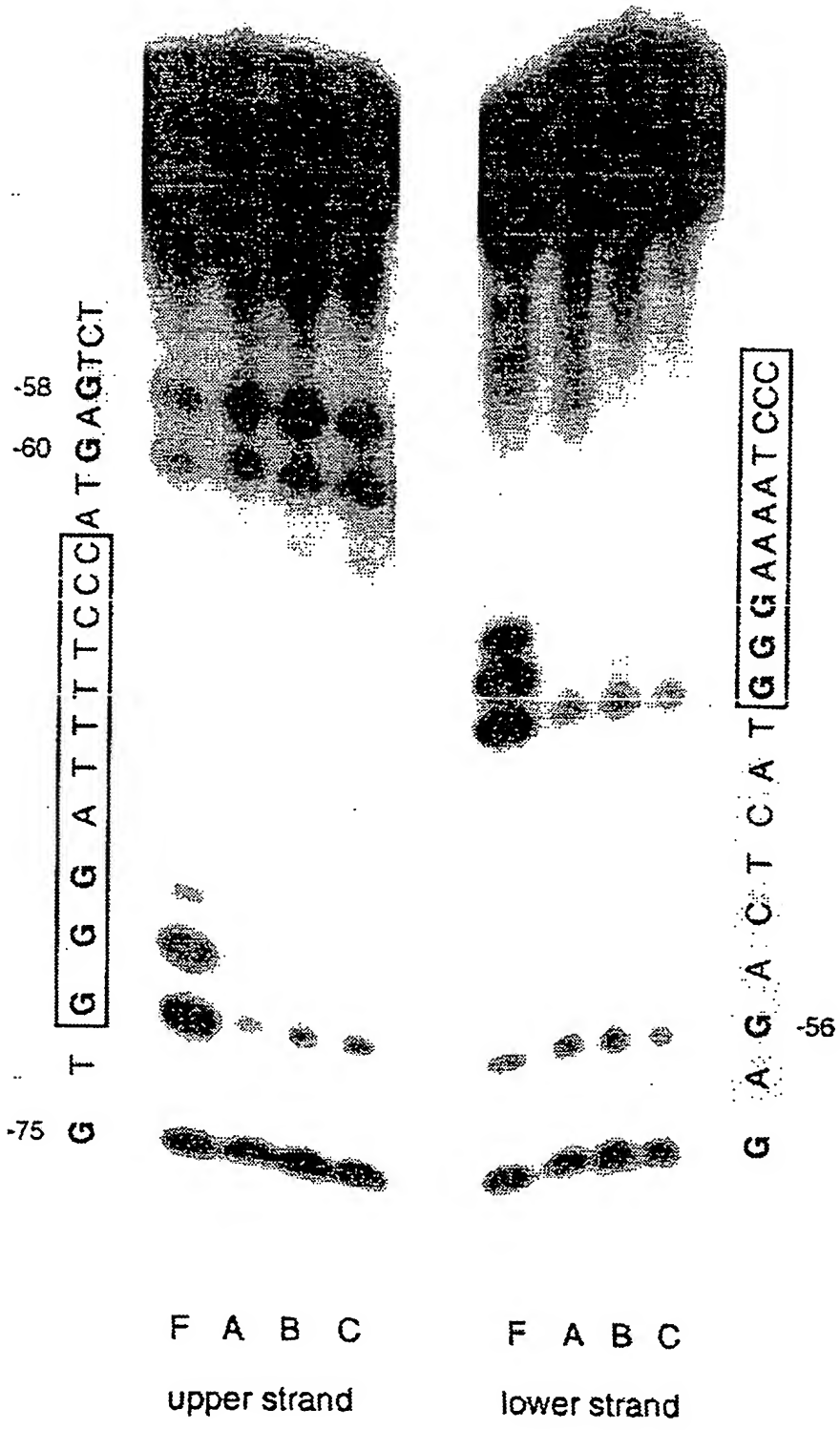
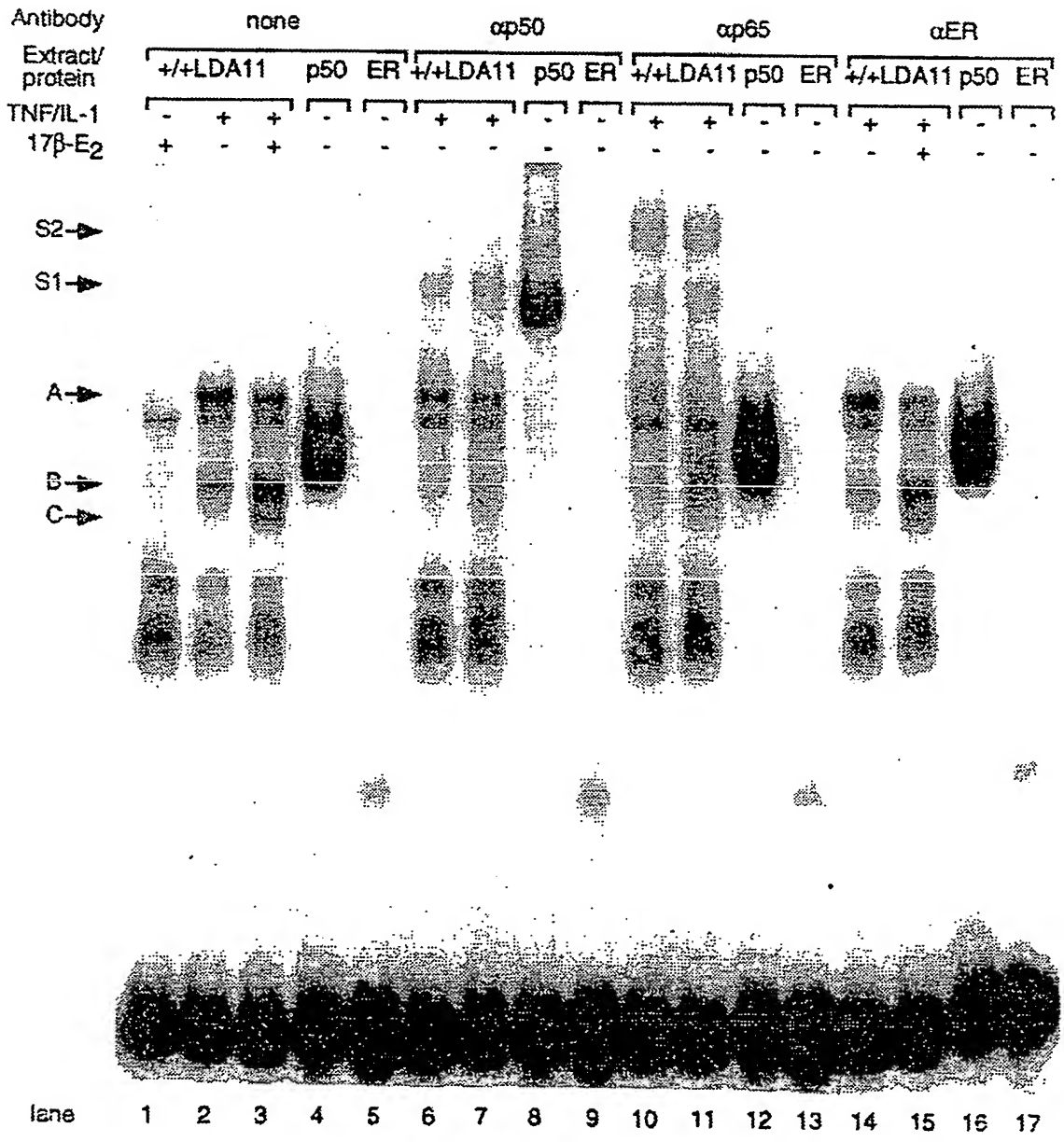


FIG. 5b.



C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. IMMUNOL., vol. 142, no. 9, 1 May 1989 pages 3134-3139, S. S. TABIBZADEH ET AL. 'Cytokine-induced production of IFN-B2/IL-6 by freshly explanted human endometrial stromal cells.' cited in the application	
P,A	MOLECULAR ENDOCRINOLOGY, vol. 9, no. 4, April 1995 pages 401-412, E. CALDENHOVEN ET AL. 'Negative cross-talk between RelA and the Glucocorticoid receptor: a possible mechanism for the antiinflamatory action of glucocorticoids. '	

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